

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Sinan Tas and Oktay Avci
Application No.:	10/682584
Filed:	October 9, 2003
For:	USE OF CYCLOPAMINE IN THE TREATMENT OF BASAL CELL CARCINOMA AND OTHER TUMORS
Examiner:	James Anderson
Group Art Unit:	1614

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DECLARATION OF DR. SINAN TAS

SINAN TAS states:

I. Background and Expertise

1. I am one of the inventors of the above titled application. I am a Medical Doctor (M.D. Hacettepe University Ankara, Turkey, 1976). I have a Ph.D. in Experimental Pathology (University of California at Los Angeles, 1980). I have also received additional educational certificates and diplomas detailed in my Curriculum Vitae, attached as **Exhibit A**.

2. My professional experience includes numerous clinical and teaching positions at academic institutions in the United States, Turkey and Kuwait working in the fields of pathology, biochemistry, clinical biochemistry, and molecular genetics. These positions are also listed in **Exhibit A**.

3. In the course of my work I have assisted in developing a first year medical curriculum at İnönü University, Malatya, Turkey and Ph.D. programs in oncology and

biochemistry at Dokuz Eylül University, Izmir, Turkey. I have also done extensive research in the fields of molecular and cellular mechanisms of aging, the structure and regulation of the eukaryotic genome and in tumor biology. I have numerous publications in those fields which are also listed in **Exhibit A**.

4. Based on my training and experience I have specific expertise in the molecular and cellular mechanisms of aging and of tumorigenesis and I am qualified to evaluate the state of the art discussed herein.

II. Level of Skill in the Art

A. Derivatives of Cyclopamine

5. Cyclopamine had long been known in prior art and identified originally due to its teratogenic effects. Teratogenicity of cyclopamine and of the related teratogenic Veratrum alkaloids had been found in prior art to arise from the inhibition of Hedgehog/Smoothed (Hh/Smo) signaling by them which resulted in the inhibition of differentiation of a group of cells in the developing embryo as a consequence (see references cited on page 3, lines 5-6 of specification). A wealth of information was publicly available in prior art about cyclopamine and about the related teratogenic compounds, including about their molecular structure, physical and chemical properties and about how to obtain them by purification from natural sources or through total chemical synthesis [see e.g. *Keeler RF, Teratology* 1970; 3:169-173 (**Exhibit B**); *Kutney JP et al, Canadian Journal of Chemistry* 1975;53:1796-1817 (**Exhibit C**); *Browne CA et al, Journal of Chromatography* 1984;336:211-220 (**Exhibit D**)]. Structure/function correlations (structure - activity relationships) were also described in prior art with the teratogenic Veratrum compounds as well as with compounds derived from them (see e.g. **Exhibit B** and *Beachy PA, WO 01/27135*, page 94, line 15 to page 98, line 22).

6. The synthesis of various derivatives of a pharmaceutically active molecule, as well as the generation of large libraries of related molecules based on modifications of the constituent structure, can be readily accomplished by modern methods of organic chemistry (e.g.

combinatorial chemistry). *Dudek et al*, US 6,291,516, recognizes this fact (see column 60, line 51 to column 61, line 42). The specification of present application indicates that molecules can be derived from cyclopamine or synthesised in such a way that they possess structural features to exert similar receptor binding properties and biological effects as cyclopamine. Such a molecule is said to be referred to as a "derivative of cyclopamine" in the specification and further information is provided about it (see page 10, lines 8-26). Such a compound is described to bind specifically to the same biological target as cyclopamine to exert the same biological effects as cyclopamine. Said effects, according to the specification, include the inhibition of Hh/Smo signaling and the causation of the technical effects recited in claim 1, when such a molecule is used according to the described method. Armed with such a clear functional definition, the skilled person can readily screen a huge number of molecules by looking for the described desirable functional effect. Assays for Hh/Smo signaling as well as for apoptosis and markers of differentiation were well known in art and widely available. High throughput versions of such assays, allowing screening together of large chemical libraries, were also known and used in prior art.

B. Tumor Treatment Methods

7. A person skilled in the art of tumor treatment has experience in routine clinical practice with conventional cancer drugs, which involves the adjustment of dosage according to the characteristics of tumor and of the patient and the expected effects of particular drugs. When informed of what effects a drug will produce, the skilled practitioner can readily optimize the administration of medicament for different tumors and patients. In this respect, the methods for determining the occurrence of apoptosis as well as of the markers of differentiation have been well known in art (they are commonly a part of the routine clinical laboratory practice). A decrease of size or disappearance of a tumor in response to an administration can also be accurately determined by the imaging methods routinely employed in clinical practice. What was not known, prior to our invention, was to look for tumors which utilize hedgehog/smoothened signaling for inhibition of tumor cell differentiation and for inhibition of tumor cell apoptosis and to develop dosages of cyclopamine or cyclopamine derivatives which go beyond inhibition of

proliferation, to achieve differentiation and apoptosis of the tumor cells. In summary, all of the methods needed to practice the invention were well known, except for the information of what effects were to be expected and therefore targeted for dosing.

III. Specification Enablement - Non-BCC Tumors

8. In an Office Action mailed September 11, 2006 in our US Patent application identified above, the Examiner has asserted that while the application is enabling for administration of cyclopamine to BCC (basal cell carcinoma) tumors, it is not enabling for inducing cellular differentiation and/or apoptosis in all tumors by administering cyclopamine. I wish to emphasise that the presently claimed method is not directed to any tumor or any tumor cell. Rather, it is directed to a specifically defined type of tumor, that is, a tumor in which Hh/Smo signaling is utilized for inhibition of tumor cell differentiation and/or for inhibition of tumor cell apoptosis. In my opinion the application does teach the skilled person how to make and use the invention for this class of tumors, without undue experimentation.

9. The specification of the present application describes a method of treatment for a specifically defined group of tumors and shows that it acts, unlike the conventional methods of drug and radiation treatments, by inductions of tumor cell differentiation and apoptosis in a highly selective, efficient and non-genotoxic manner. The specification describes and exemplifies successful applications of the method on several independent tumors (>7) and on more than one type of tumor that qualify for the specific definition given in claim 1. The method was successful each time it was applied and not only on BCC's but also on the other types of tumors within the defined category (e.g. trichoepithelioma and melanocytic tumors). All of the exemplified tumors were human tumors. The described examples are highly relevant to the practice of invention clinically. Actual human patients bearing the tumors were treated to attain the technical effects and therapeutic results that are described in specification. Microscopic views of the tissue sections are shown that show that the new method of treatment causes a "massive apoptotic death and removal of tumor cells" so as to cause "disappearance of tumor nodules to leave behind cystic spaces with no tumor cells." All of this is shown to be achieved

while sparing normal tissue cells (including those with stem and progenitor cell features). In addition to the clinical photographs and microscopic views showing the therapeutic results and technical effects, statistical analyses are also shown which document the reproducibility and efficiency of the inductions of tumor cell differentiation and apoptosis by the new method of treatment (see page 21, Table 1). The skilled person will realize that a statistical analysis of the described treatment results (successful outcome in each of >7 independent tumors) also shows a high predictability of the method on the specific type of tumor and tumor cells described in the pending claims.

10. A person skilled in the art is also guided by the specification in practicing the invention on other tumors that qualify as described in the pending claims. The demonstration that the qualified tumor cells can be caused to undergo differentiation and apoptosis *in vivo* with the described efficiency and selectivity, by administration of a sufficient dosage of medicament, is by itself highly informative to the skilled person. The specification teaches conditions providing the described technical effects with independent exemplified tumors (page 4, line 22 to page 6, line 9; page 7 line 1 to page 8, line 27; page 10 line 28 to page 14, line 2; page 19, line 4) and the skilled person is reminded that the optimal quantity and application schedule of medicament depends on the size and location of tumor as well as other factors that are well known in art.

11. The specification of our application describes that the use of cyclopamine and cyclopamine derivatives can be used in treatments of BCC (basal cell carcinoma) and non-BCC tumors so as to cause a rapid regression or disappearance of tumors that employ the Hh/Smo signaling for inhibition of apoptosis and/or for inhibition of differentiation of tumor cells. Information on specific non-BCC tumors is provided on page 7, line 21 to page 8, line 4; page 16 line 22 to page 17 line 4; page 17 lines 4-11; and Figures 6A, 6B, 6C, 6D. Further, the specification teaches that the results obtained with the exemplified tumors, including the sparing of normal stem and progenitor cells, indicate effectiveness in applications for internal tumors that also employ Hh/Smo signaling for inhibition of apoptosis and for inhibition of differentiation of tumor cells (e.g. the provision of guidance with regard to the doses when medicament is given by

systemic or intratumoral administration) (see page 11, lines 22-27; page 15, lines 19-24; page 19, lines 5-10; page 19, lines 12-29). Further relevant in this regard is the fact that patched 1 haploinsufficiency had been well recognized in prior art to cause predisposition to not only BCC's but also to other tumors in various organs and tissues of affected people. As noted in the specification (page 2, lines 13-16), this condition is called Nevoid Basal Cell Carcinoma Syndrome (NBCCS or Gorlin's syndrome). It was well known in prior art that, besides BCC's, occurrences of numerous other benign and malignant tumors (including medulloblastoma, fibroma, fibrosarcoma, meningioma, lymphoma, rhabdomyoma and schwannoma) are increased in the patients with NBCCS. For example, recurrent independent non-BCC tumors were described in many NBCCS patients and ovarian fibromas were found in 17% of the female NBCCS patients (*Kimonis VE et al, American Journal of Medical Genetics* 1997;69:299-308) (**Exhibit E**). Still further, the loss-of-function mutations of patched (i.e., the underlining cause of NBCCS) had been well known in prior art to cause upregulation of Hh/Smo signaling and were described also in the sporadic BCC's and various other sporadic tumors, including medulloblastoma, meningioma, breast cancer, uterine cervix and esophageal squamous cell carcinomas and trichoepithelioma [*Xie J et al, Cancer Research* 1997;57:2369-2372 (**Exhibit F**); *Vorechovsky I et al, Cancer Research* 1997;57:4677-4681 (**Exhibit G**); *Maesawa C et al, Genes Chromosomes Cancer* 1998;21:276-279 (**Exhibit H**); *Lu X et al, International Journal of Gynecological Cancer* 2000;10:452-458 (**Exhibit I**)]. Thus, a person skilled in the art, after reading the specification, understands readily that the described method can be utilized for treating not only BCC's but also other tumors that also qualify for the definition given therein.

12. Table 1, below, identifies teachings in our specification which indicate that the described method of treatment has utility in treatment not only of the BCC's but also of all those specific tumors that employ the Hh/Smo signaling for inhibition of apoptosis and/or for inhibition of differentiation of tumor cells.

Table I. Teachings In Specification That The Claimed Method Of Treatment Can Provide The Technical Effects Described In Claim 1 On The Non-BCC Tumor Cells That Also Employ The Hh/Smo Signaling For Inhibition Of Apoptosis And/Or For Inhibition Of Differentiation

Page 1, lines 1-2	" ... treatment of basal cell carcinoma and other tumors "
Page 3, lines 24-28	" These novel effects ... make ... highly desirable in cancer therapy, in the treatment of BCC's and other tumors that use the hedgehog/smoothened signal transduction ... for ... prevention of apoptosis"
Page 4, lines 12-17	" In a further aspect, the invention is directed to the use of cyclopamine or ... a derivative of cyclopamine in the treatment of tumors that use the hedgehog/smoothened signal transduction ... for the prevention of apoptosis or ... differentiation "
Page 7, lines 21-25	" ... trichoepithelioma ... prior to treatment ... after 24 hours of treatment "
Page 7, line 27 to page 8, line 4	" ... section ... numerous apoptotic cells and the formation of cystic structures by their removal "
Page 9, line 22 to page 10, line 4	" ... tumor cells ... cyclopamine-induced differentiation ... apoptosis ... highly efficient and ... extreme specificity ... make it highly desirable in cancer therapy "
Page 11, lines 22-27	" Preservation of the undifferentiated cells ... provide information about the tolerable doses in other ... modes of administration ... intratumoral injection of an aqueous solution or systemic administration of the same or of cyclopamine entrapped in liposomes "
Page 15, lines 19-24	" ... highly efficient differentiation and apoptosis of the tumor cells in vivo ... at doses that preserve the undifferentiated tissue cells ... together with the non-genotoxic mode of action ... support the use ... not only on BCC's but also on those internal tumors that utilize the hedgehog/smoothened pathway for ... prevention of apoptosis and/or differentiation "
Page 16, line 22 to page 17, line 4	" Trichoepithelioma is another tumor associated with genetic changes that cause increased hedgehog/smoothened signalling ... rapid regression ... marked apoptotic activity ... cystic spaces resulting from the apoptotic removal of tumor cells "
Page 17, lines 4-11	" ... decreased size and pigmentation ... melanocytic tumor ... treatment ... in another volunteer also caused similarly rapid ... disappearance "
Page 19, lines 2-10	" ... tumor response to optimal concentrations ... was rapid ... examples illustrate effectiveness of the described treatment in the causations of tumor cell differentiation and apoptosis and in ... regression of the tumors displaying hedgehog/smoothened signaling. Effectiveness on several independent tumors in unrelated patients ... is consistent with the general utility "
Page 19, lines 12-29	" ... findings that a transient exposure to cyclopamine can suffice for the causations of tumor cell differentiation and apoptosis ... further ... facilitates treatment of internal tumors "
Page 22, line 1 to page 24 line 23	Originally filed claims 1-3, 5-14, 16-22 directed to a method employing cyclopamine or a pharmaceutically acceptable derivative thereof or to a medicament comprising the same for obtaining the technical effects described in claim 1 on the specific tumor cells defined therein.

13. Independent scientific investigations published after our invention confirm that cyclopamine or a derivative thereof can be utilized for obtaining the technical effects recited in claim 1 not only on BCC but also on the non-BCC tumor cells as described in claim 1. Such publications include *Berman DM et al, Science* 2002; 297:1559-1561 (**Exhibit J**), *Thayer SP et*

al, *Nature* 2003;425:851-856 (**Exhibit K**) and *Sanchez P et al*, *Mechanisms of Development* 2005;122:223-230 (**Exhibit L**). These published investigations include tumors located internally and a systemic mode of administration. The methods of treatment in these investigations are fully consistent with that described in the present application. For example *Berman DM et al* (**Exhibit J**), experimenting with medulloblastoma, describe inductions of tumor cell differentiation and apoptosis by use of cyclopamine and report that systemic administration of cyclopamine to tumor-bearing mice at a dosage of ~ 50 mg/kg body weight daily for 24 days resulted in the disappearance of established tumors. Similarly, *Thayer SP et al* (**Exhibit K**) describe that systemic administration of cyclopamine to the mice having pancreatic adenocarcinoma allograft at a dosage of ~ 48 mg/kg body weight daily for 7 days induced marked apoptotic death of tumor cells. In comparison, in the tumor treatment described by *Dudek et al*, who described an inhibition of tumor cell proliferation but did not describe induction of tumor cell apoptosis, the cyclopamine dosage given systemically to the medulloblastoma-bearing mice was magnitudes lower (1.1 mg/kg body weight daily for 14 days). In accordance, *Sanchez P et al* (**Exhibit L**) reported that systemic administration of cyclopamine to medulloblastoma-bearing mice at a dosage of 10 mg/kg body weight every other day for a month did not suffice to cause detectable tumor cell apoptosis although tumor cell proliferation was inhibited. They report further that cyclopamine can be used for inducing tumor cell apoptosis but the causation of this technical effect requires relatively greater doses than that is sufficient to inhibit tumor cell proliferation.

14. Regarding the assertion on pages 5-6 of the Office Action, that *Sanchez et al*, (**Exhibit L**), demonstrates that inducing differentiation and/or apoptosis is highly unpredictable, I do not agree that it would be viewed as such by a skilled person who has been given access to our application. *Sanchez et al*, confirms what is taught in the specification of present application, namely, induction of apoptosis of the tumor cells that are defined in claim 1 is dependent on the quantity and duration of application of cyclopamine. See page 228, second column: "Indeed, apoptosis driven by Hh pathway blockade is often more evident in vitro after a prolonged period of treatment or at high doses (data not shown)." It does not follow from this fact that the attainment of said technical effect on the specific tumor cells described in claim 1 is

unpredictable. Rather, it demonstrates that the application is correct in asserting that the dosage must be high enough to produce more than inhibition of proliferation.

15. Other scientific publications describing administration of cyclopamine onto tumor cells in vitro and the Hh/Smo signaling characteristics in various human tumors also confirm that the presently claimed method of treatment has utility in treatments of the specific tumors described in this application. Thus, *Mimeault M et al (International Journal of Cancer 2006; 118:1022-1031) (Exhibit M)* describe that cyclopamine can be utilized to induce apoptosis of the human prostate cancer cells in a concentration-dependent manner (see *e.g.* pg. 1025, col. 2).

16. *Sheng T et al, Molecular Cancer 2004;3:29 (Exhibit N)* provide further confirmation that the skilled person can identify non-BCC tumors encompassed within claim 1. Reporting findings with 59 human prostate cancer specimens, they describe that the prostate cancers that had a relatively greater proportion of tumor cells displaying features of differentiation had lower Hh/Smo signaling activity than the undifferentiated prostate cancers. Similar confirmatory evidence (i.e., the utilization of Hh/Smo signaling in inhibition of tumor cell differentiation) have been described independently with human uterine cervix squamous cell cancers and precursor lesions (*Xuan YH et al, Modern Pathology 2006;19:1139-1147) (Exhibit O)* and with ameloblastomas (*Kumamoto H et al, Journal of Oral Pathology and Medicine 2004;33:185-190) (Exhibit P)*. Also, *Oliver TG et al [Development 2005;132:2425-2439 (Exhibit Q)]* have described an analysis of the medulloblastoma development in the mice born, like the human NBCCS patients, with haploinsufficiency of patched 1. They have demonstrated that, whereas both the medulloblastoma cells and nontumorigenic granule cell precursors show proliferation, the tumor cells, but not the non-tumorigenic cells express the Hh/Smo signaling target genes whose products act to inhibit differentiation and apoptosis in tumor cells.

IV Non-obviousness over Dudek et al, US 6,291,516

17. I have read the disclosure of *Dudek et al* repeatedly and the comments regarding

this document in the Office Action. I do not agree that a skilled person, is led to the present invention by anything taught in this document. In this regard it is important to recognize that much of the disclosure in *Dudek et al* is so generalized and unsupported as to be useless. The patent purports to describe "regulators" of the hedgehog pathway in which an unlimited number of compounds are said to antagonize hedgehog signaling. In addition, another category of unlimited number of compounds are disclosed that may function to increase or decrease the intracellular cAMP concentration ¹. *Dudek et al* refers to all of these diverse compounds as "subject compounds", regardless of the fact that they have opposing regulatory actions. Further, a widely diverse range of conditions are indicated to be affected by these "regulators." Consequently, except when a clarification is made (or can be inferred) as to the nature of "subject compounds" to be used for a given condition, it is typically not clear which of the compounds are to be used for which condition and what regulatory effect may be expected.

18. Many of *Dudek et al*'s teachings lead away from the use of cyclopamine or another specific inhibitor of hedgehog signaling for induction of cellular differentiation. In Table II below, I have detailed teachings in *Dudek et al* which specifically would have lead away from use of cyclopamine or another specific inhibitor of hedgehog signaling for induction of differentiation.

Table II. Unambiguously Disclosed and/or Supported Teachings in *Dudek et al* That Teach Away From The Use Of Cyclopamine Or Another Specific Inhibitor Of Hedgehog Signaling For Induction Of Cellular Differentiation.

<u>Column and Lines</u>	<u>Teaching</u>
Col. 2, lines 5-7	"... critical inductive roles of hedgehog polypeptides in the development "
Col. 3, lines 16-17	" In the CNS, Shh ... induce ventral cell fates " (references are given)
Col. 3, lines 44-45	" ... Shh also induces the appropriate ventrolateral neuronal cell types " (references are given)
Col. 3, lines 60-61	" ... Shh mediates the induction of the sclerotome directly " (reference is given)

¹ Compounds that cause increase of intracellular cAMP are said to be used to inhibit hedgehog signaling. Whereas activation of the protein kinase A (PKA) by increased intracellular cAMP is known to be capable of inhibiting hedgehog signaling, it must be emphasized that the increases of intracellular cAMP and of PKA activity can have many other consequences in the cell besides the affection of hedgehog signaling. For example, many polypeptide hormones are known to act by causing elevation of intracellular cAMP. Further, the PKA activity is well known to affect Wnt/ β -catenin signaling besides hedgehog signaling. It is clear that the actions of the compounds disclosed to alter cAMP concentration would not be specific to hedgehog signaling.

Col. 42, lines 61-63	" Shh serves as an inductive signal ... which is critical "
Col. 43, lines 22-26	" ... differentiation of intestinal mesoderm ... depend on ... Sonic hedgehog " (reference is given)
Col. 48, lines 42-45	"... a subject compound can reduce the ... proliferation and differentiation of ... epithelium " (subject compounds that cause inhibition of proliferation are taught in <i>Dudek et al</i> to be compounds that inhibit hedgehog signaling)
Col. 49, lines 31-33	" Ihh ... promote retinal progenitor proliferation and photoreceptor differentiation " (reference is given)
Col. 49, lines 34-39	"... treatment ... with ... Sonic hedgehog results ... in rod photoreceptors, amacrine cells and Muller glial cells " (reference is given)
Col. 67, lines 40-49	" Skin punches treated with ... Shh ... display ... follicle induction ... Shh leads to significantly increased induction "

19. *Dudek et al* teach also directly away from the use of subject compounds for induction of apoptosis. Referring to the requirement of proliferation for the induction of cell death by many agents used in treatment of cancer, *Dudek et al* disclose specifically that "*because a subject compound will often be cytostatic to epithelial cells, rather than cytotoxic*", subject compounds can be used to inhibit proliferation and to prevent thereby the programmed cell death in hair follicles (column 50, lines 20-29).

20. *Dudek et al* has no teaching that cyclopamine or another specific inhibitor of hedgehog signaling will induce apoptotic death and removal of tumor cells so as to cause a decrease of size or disappearance of tumor. Rather, *Dudek et al*'s teachings point in the opposite direction, as elaborated below.

21. *Dudek et al* had actually evaluated cyclopamine as well as another specific inhibitor of hedgehog signaling (jervine) on the tumor cells that had constitutive activation of hedgehog signaling due to an inactivating mutation in the gene for patched. They describe the *in vitro* findings in column 63, lines 13-50 and in Figure 2 and disclose that both cyclopamine and jervine inhibited the proliferation of said tumor cells. No causation of cell death by cyclopamine or jervine is disclosed even though the treated cells were examined by microscopy.

22. Cyclopamine was evaluated by *Dudek et al* also *in vivo* on the same tumor (medulloblastoma) cells. The related experiments and findings are described in column 63, line 51 to column 64, line 22 and in Figure 3. Contrary to the mistaken statement on page 12 of the September 11, 2006 Office Action, *Dudek et al* do not describe, and the disclosed experiments and findings do not show, causation of a decrease of medulloblastoma tumor volume by the described cyclopamine treatment. *Dudek et al* describe that they established transplanted tumors in athymic mice by injecting medulloblastoma cells into their brain and by allowing 5 weeks for the tumors to grow. The tumor-bearing mice were then divided into two groups for treatment during the next 2 weeks with injections of cyclopamine (3 mice) or vehicle (2 mice). One of the three mice injected with cyclopamine was sickened during the course of injections and had to be killed before the end of 2 weeks. All remaining animals were killed at the end of the 2 weeks of treatment and the volumes of existing tumors were determined. Figure 3 shows the individual tumor volumes in arbitrary units (relative tumor volumes). The volume of the tumor from the cyclopamine-treated mouse that was killed earlier than the others is included inappropriately in the data set shown in Figure 3, with no indication of which data point belongs to said mouse. Whereas the cyclopamine-treated group had an average tumor volume smaller than in the vehicle-treated group, the difference was not significant ($p > 0.05$, calculated from the data set shown in Figure 3). No causation of cell death by the treatment with cyclopamine is disclosed even though serial sections of tumors were made and examined by microscopy. Such microscopic examinations ordinarily enable detection of apoptosis as shown for instance in the Figures of our application. In addition, design of the experiments described in *Dudek et al* is lacking a measurement of the volumes of growing tumors prior to the initiation of treatments (i.e., at the end of the 5th week). *Therefore, even in case of a significant difference between the vehicle and cyclopamine treated groups in terms of tumor volumes, these experiments would only permit a conclusion of an inhibition of tumor growth by the cyclopamine treatment.*

23. *Dudek et al* teaches that that a minimum dosage that will produce a therapeutic effect is to be used (column 54, lines 42-49; " ... start ... at levels lower than that required ... and gradually increase the dosage until the desired effect is achieved"). What is contemplated in terms of tumor treatment is an inhibition of tumor growth (see column 64, lines 10-12), and what

is shown in *Dudek et al* in terms of the technical effect obtained on tumor cells by use of cyclopamine is an inhibition of proliferation. Thus the manipulations in the *Dudek et al* method involve an administration in a quantity that will produce the technical effect of the inhibition of tumor cell proliferation, but not more. It is specifically taught in *Dudek et al* not to administer beyond that quantity.

24. Our application describes a clear dose-response effect in the attainment of differentiation and/or apoptosis and emphasizes that a suboptimal dosing would not provide said technical effects (see e.g. page 18 line 21 to page 19 line 4 and Figure 7F).

25. Some of the independent investigations already mentioned which confirm the scope of the invention also report that tumor cell apoptosis is not obtained until a threshold dosage is exceeded that is above the dosage sufficient to inhibit tumor cell proliferation. Thus, *Sanchez P et al* (**Exhibit L**) describe that an amount or dosing of cyclopamine that caused inhibition of tumor cell proliferation did not cause a detectable tumor cell apoptosis and that the causation of latter technical effect required relatively higher doses. Similarly, *Mimeault et al* (**Exhibit M**) found with cell lines of human prostate cancer cells that induction of tumor cell apoptosis requires relatively greater amounts of cyclopamine and longer durations of treatment than needed to obtain an inhibition of proliferation of the same tumor cells. For example, it is described that treatment of the LNCaP-C33 prostate cancer cells with 2 μ M cyclopamine for two days caused inhibition of tumor cell proliferation (see Fig. 2 of **Exhibit M**) but even a treatment with more than twice of that amount for four days failed to cause detectable apoptosis (**Exhibit M**, Fig. 4A, 5 μ M Cycl). Apoptosis, however, is very clearly induced after treatment of said cells with 20 μ M cyclopamine for four days (**Exhibit M**, Fig. 4A, 20 μ M Cycl).

26. Besides the above mentioned evidence and facts, it should be noted that the design of the investigations described in the specification of this application entails medicament applications onto a part of tumor in a patient, followed by the analyses of responses in entire tumor. In such an approach, the tumor area that is adjacent to the area onto which cyclopamine is applied receives a relatively lower dosage of cyclopamine by diffusion from the area of direct

application. Thus, the effects of differing amounts of cyclopamine on tumor cells can be assessed with the cells of same tumor *in vivo*. The specification, in this respect, refers to the results of such investigations and emphasizes that a suboptimal dosing would fail to provide the technical effects described (i.e., the inductions of tumor cell differentiation and apoptosis; see e.g. page 18, line 21 to page 19, line 4). The tumor depicted in Figures 5A-F of specification is a tumor treated in such a manner and shows most directly that a dosage that suffices to inhibit tumor cell proliferation *in vivo* in a patient does not suffice to induce tumor cell apoptosis. The tumor of Figure 5A was treated by topical application of cyclopamine on half of its surface (the lower half in figure) whereas the other half (upper in figure) was left untreated. After treatment (Figure 5B) the entire tumor was removed and sectioned. Figures 5C-5F of the application depict various portions of the tumor stained by H&E or immunohistochemically for Ki-67 as described therein. Sections stained for Ki-67 are reproduced in color below as Figures 1A and 1B, at a relatively lower magnification than in the Figs 5E and 5F of application in order to show more tumor depth.

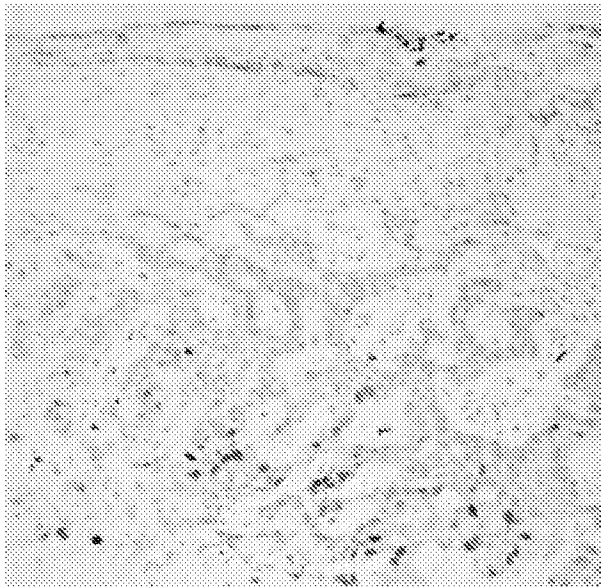


Figure 1A

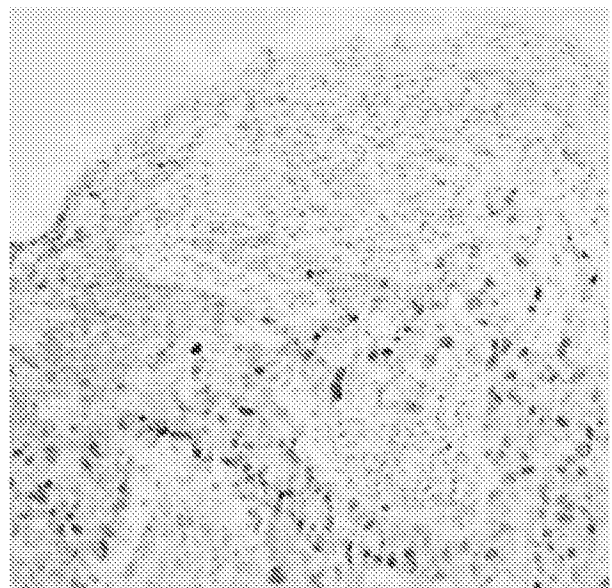


Figure 1B

Figure 1A: Section from the basal cell carcinoma shown in Fig. 5B of the specification. This section is from the half of tumor onto which the medicament was applied directly as described in the

specification. Immunohistochemical staining for Ki-67 (which is known to be a marker of proliferating cells) was done as described in the specification. The brown-labeled nuclei represent the proliferating cells (100 X original magnification).

Figure 1B: Section from the basal cell carcinoma shown on Fig. 5B of the specification. This section is from the half of tumor onto which cyclopamine was not applied directly. However, because of being in close proximity, a relatively lower concentration of cyclopamine than that in the adjacent area of direct application would reach to this area by diffusion from the area of direct application. Ki-67 staining (100 X original magnification).

27. Comparison of Figures 1A and 1B above shows that a relatively low dose of cyclopamine that does not exceed what is sufficient to inhibit tumor cell proliferation would fail to provide tumor cell apoptosis. The brown labeled tumor cell nuclei represent the proliferating tumor cells. In both Figures 1A and 1B, orientation of the tumor section is such that the tumor surface is towards the upper part of figure whereas the bottom of figure shows deeper regions of tumor. In both figures, the regions of tumor near the tumor surface show lack of Ki-67 labeling (i.e., the cells show inhibition of tumor cell proliferation) due to the cyclopamine that diffused down from the surface where it was applied directly (Figure 1A) or from the adjacent direct application area (Figure 1B). The deeper tumor regions show proliferating (Ki-67 labeled) tumor cells but the frequency of labeled tumor cells is relatively higher in the deeper tumor regions in Figure 1B than in Figure 1A. Notice in Figure 1A that the region of tumor near surface shows extensive formation of cystic spaces within the tumor. This is because the tumor cells therein were killed by apoptosis and removed to leave behind (empty) cystic spaces. By contrast, in Figure 1B the cell density is much higher in the tumor region towards the surface. In addition, the large cystic spaces that are seen in Figure 1A within the tumor are not visible in Figure 1B in the region where tumor cell proliferation was inhibited. Tissue sections that are stained for Ki-67 by immunohistochemistry are not suitable for quantification of apoptosis but H&E stained tumor sections are known to allow identification of the tumor cells having morphological features of apoptosis. For example, the H&E stained section in Fig 5C of specification shows numerous readily identifiable apoptosing tumor cells and demonstrates that the cystic spaces seen in the tumor are being formed as a result of the apoptotic death and removal of tumor cells. Counting apoptosing tumor cells in a microscopic field of an H&E stained section together with the total number of tumor cells allow one to obtain a measure of the frequency of apoptosing tumor cells therein. Said measurements can be repeated in other randomly sampled microscopic fields

belonging to a particular region of tumor so as to obtain a mean and a standard error of mean. Similar measurements of the Ki-67 labeling frequency in serial tumor sections (one stained for Ki-67 and next by H&E) thus allow one to measure the frequencies of proliferating and apoptosing tumor cells in a given region of tumor. This would provide an insight into the relative effects of the same concentration of cyclopamine on the proliferation and survival/apoptosis of tumor cells in the same tumor region *in vivo*.

28. I have done a quantitative comparison of the tumor cells showing proliferation and of the tumor cells showing apoptosis at three depths in the tumor shown in Fig 5B of specification. Graphic representation of my results are shown below in Figures 2A and 2B.

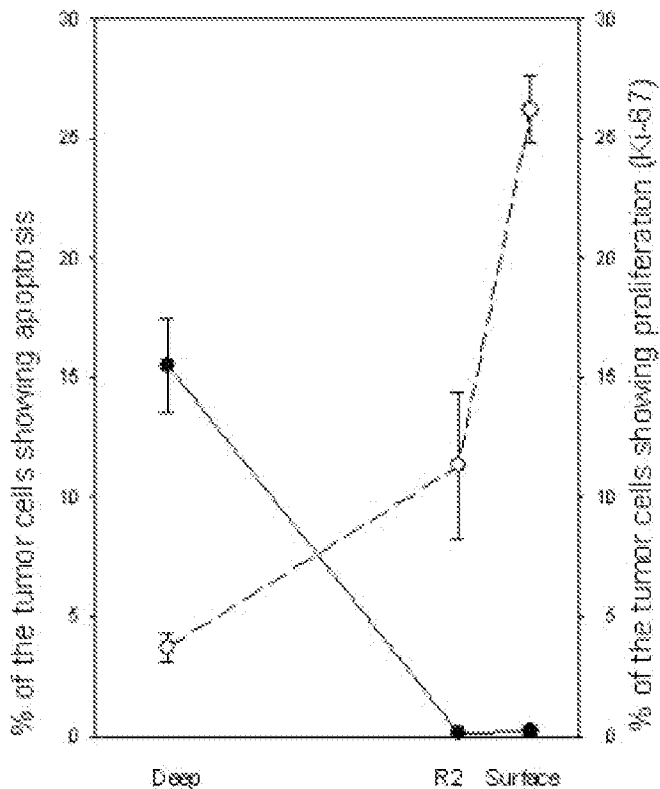


Figure 2A

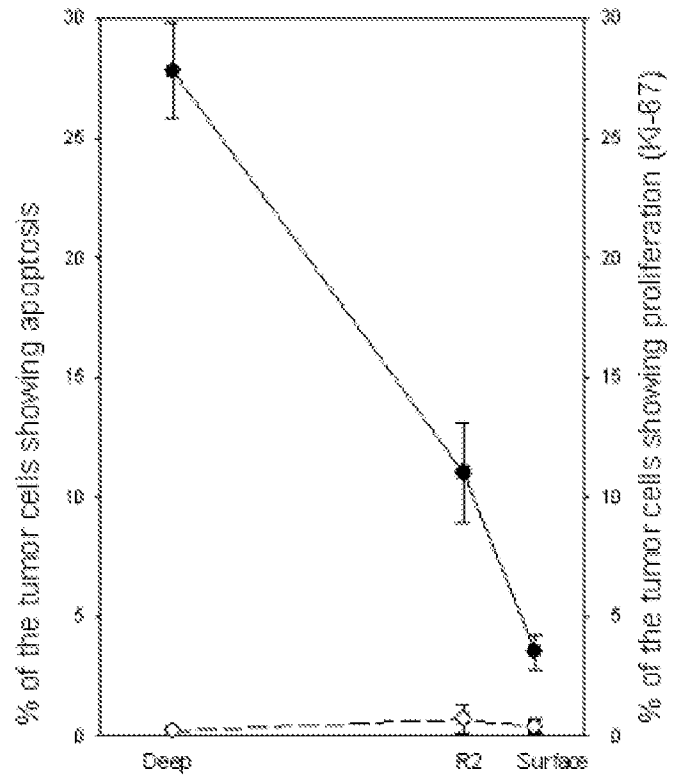


Figure 2B

Figures 2A and 2B show quantitative data concerning the frequencies of the proliferating tumor cells as well as of the apoptosing tumor cells in the different regions of the tumor shown in Fig. 5B of specification. Figure 2A shows data from the half of tumor where cyclopamine was applied directly. Figure 2B shows data from the half of tumor where cyclopamine could reach by diffusion from the adjacent direct application area. On the X axis, "Deep" refers to the region of tumor deep in dermis,

near the invading border. "Surface" refers to the surface region of tumor (i.e., the region that would receive the highest concentration of cyclopamine). "R2" refers to the region adjacent to the surface region. The scale on the X axis corresponds to the position of the analyzed regions in terms of depth from the tumor surface. The symbols (• — •) represent the frequency of Ki-67 labeled (i.e., proliferating) tumor cells. The symbols (o ... o) represent the frequency of tumor cells showing morphological signs of apoptosis.

The data in Figure 2A are from the half of tumor onto which cyclopamine was applied directly. The data in Figure 2B are from the half of tumor onto which cyclopamine was not applied directly; a relatively lower concentration of cyclopamine would have reached to this half by diffusion from the adjacent half onto which cyclopamine was applied directly. In addition, in both Figures 2A and 2B, the tumor cells near the surface (labeled as "Surface" on the horizontal axis in graphs) would have received greater dosage of cyclopamine than those tumor cells located deep in tumor (labeled as "Deep" on the horizontal axis in graphs). On the graphs, the symbols (• — •) represent the frequency of proliferating tumor cells and the symbols (o o) represent the frequency of tumor cells showing features of apoptosis (the bars on the two sides of symbols represent the standard errors). The scale on the horizontal axis corresponds to the position of analyzed regions in terms of depth from surface. Notice that the highest frequency of proliferating tumor cells was found in the tumor region ("Deep" in Figure 2B) that is furthest away from the site of cyclopamine application. Notice also that relatively higher doses of cyclopamine are required for the induction of tumor cell apoptosis than that would be sufficient to inhibit tumor cell proliferation. These quantitative data demonstrate clearly that stopping administration of cyclopamine at a dosage that suffices to inhibit tumor cell proliferation would not produce detectable apoptosis of tumor cells. Hence, a treatment as described by *Dudek et al* would not be able to provide detectable tumor cell apoptosis or the disappearance of an existing tumor.

29. Cyclopamine or a derivative thereof or another specific inhibitor of Hh/Smo signaling had been applied in prior art on tumor cells and on transformed cells displaying constitutive activation of Hh/Smo signaling. Table III, below, shows the prior art documents reporting results of said investigations along with their relevant teachings and show that they were leading away from the present invention. These prior art documents were previously filed in IDS submissions as indicated in the Table.

Table III. Teachings In Prior Art That Teach Away From The Use Of Cyclopamine Or A Derivative Thereof For Inducing Tumor Cell Apoptosis

Document	Filed date of IDS (document no.)	Teaching
<i>Dunn MK et al, Developmental Dynamics</i> 1995;202:255-270	5/3/06 (CAC)	" ... cell death is a specific effect of cyclopamine on (normal) cranial crest cells ... viability ... of cell line 3T3L1 or ... PC12 " are not affected (page 259, left column, 3 rd paragraph). " ... 3T3L1 and PC12 cell lines are resistant to cyclopamine-induced cell death " (page 265, right column, 2 nd paragraph). See also the Abstract and Fig. 12 (page 267) (PC12 is a well known tumor-derived and tumorigenic cell line).
WO 99/52534	1/27/04 (BA)	Treatment of patched -/- cells displaying constitutive activation of hedgehog signaling with cyclopamine caused marked inhibition of said signaling but <i>did not cause cell death</i> (page 102, lines 23-30). " ... because a hedgehog antagonist will often be cytostatic to epithelial cells rather than cytotoxic ", subject compounds can be used to inhibit proliferation and to prevent thereby the programmed death of cells (page 54, lines 1-11).
WO 01/27135	1/27/04 (BD)	Treatment of patched -/- cells displaying constitutive activation of hedgehog signaling with cyclopamine or KAAD-cyclopamine (a derivative of cyclopamine) did not cause cell death " ... even at the highest doses of compounds used " that caused inhibitions of the hedgehog signaling and of cellular proliferation. " ... because a subject compound will often be cytostatic to epithelial cells, rather than cytotoxic ", such agents can be used to inhibit proliferation and to prevent thereby the programmed death of cells (page 68, lines 11-21).
<i>Taipale J et al, Nature</i> 2000;406:1005-1009	1/27/04 (CC)	Treatment of patched -/- cells displaying constitutive activation of hedgehog signaling with cyclopamine or with KAAD-cyclopamine (a derivative of cyclopamine) caused dose-dependent inhibitions of both hedgehog signaling and of cellular proliferation but did not cause cell death (page 1007, Figure 3, legend).

30. It is clear from the documents mentioned in Table III above that cyclopamine and other specific inhibitors of hedgehog signaling were actually administered in prior art on tumor cells and on the transformed cells displaying constitutive activation of hedgehog signaling, but not to an extent that apoptosis was induced. Descriptions of none of these investigations included an induction of apoptosis; to the contrary, it was mentioned specifically that the treatments causing a complete inhibition of hedgehog signaling as well as marked inhibition of proliferation did not cause a death of the transformed and tumorigenic cells. Thus, the experimental findings described in the prior art with regard to the effects on tumor cell survival/death are simply the opposite of what is described in this invention. Not only the use of

an inhibitor of hedgehog signaling for inducing tumor cell apoptosis is surprising, but also the sparing of normal cells (particularly the normal stem and progenitor cells) under conditions causing a massive apoptosis of tumor cells is highly unexpected. *Thus, the skilled person would not even contemplate trying to find conditions where the technical effects described in our invention would be achieved.*

31. Prior to our invention, there were also other teachings in the prior art that would have led the skilled person away from the present invention. In particular:

a) Induction of tumor cell apoptosis *in vivo* in a selective and efficient manner so as to cause disappearance of tumors while sparing normal cells was considered in prior art as an unattainable goal on theoretical grounds and in view of the vast clinical experience (see e.g. the review article "*Apoptosis and the dilemma of cancer chemotherapy*"; Hannun YA, *Blood* 1997;89:1845-1853 [**Exhibit R**]).

b) A well known problem of conventional non-surgical treatments of cancer is the causation of damage to normal stem cells. Yet, these cells were described in prior art to be absolutely dependent on hedgehog signaling for their maintenance (Zhang Y et al, *Nature* 2001;410:599-604 [**Exhibit S**]).

c) Hedgehog proteins have been well known as inducers of differentiation of various cell types and the experiments where hedgehog signaling was inhibited by specific inhibitor compounds had shown inhibition of differentiation by said compounds (see e.g. the references mentioned in Table II).

d) Description of cyclopamine in prior art as a teratogen would discourage its consideration as a medicament.

32. The previously unknown technical effects of the inductions of tumor cell differentiation and apoptosis by use of a specific inhibitor of hedgehog signaling, with the efficiency and selectivity described in this invention, provided the therapeutic outcome of rapid regression and disappearance of tumors while sparing normal cells. The normal cells having the attributes of stem or progenitor cells, which are well known to be hit by most conventional cancer treatments to such an extent to limit the applicability and efficacy of such treatments, are

shown to be spared. These described features of present method clearly distinguish it from prior art methods of tumor treatment and makes it superior to them. Non-limiting examples of the clinical conditions where the presently described method of tumor treatment would be preferred over prior art methods include the following.

a) Rapid shrinking and disappearance of tumors, owing to the induction of a massive apoptotic death and removal of tumor cells, identifies a group of patients in whom surgical removal of tumor may not be feasible because of the location and/or size of tumor and in whom a mere inhibition of further tumor growth may be insufficient.

Treatment of such a patient may be attempted by conventional means, e.g. by irradiation of tumor, but this is well known to be unfeasible or inadvisable for particular tumors and patients.

b) Tumors are known to undergo decrease of size *in vivo* for reasons other than the induction of tumor cell apoptosis, for example due to the causation of necrosis.

However, removal of tumor cells by apoptosis is preferred for several reasons, including the severe inflammatory reaction that follows necrosis but not apoptosis.

c) Cessation of cellular proliferation, when it occurs because of being coupled to a certain differentiation state, would be advantageous in tumor treatment over a treatment protocol involving inhibition of tumor cell proliferation with little or no induction of differentiation. Many prior art methods of treatment belong to this latter category and the need for continuous administration of medicament for inhibition of tumor cell proliferation poses a dilemma because of the toxicities that become apparent with such administration. Cessation of tumor cell proliferation after an initial treatment, as described in this application, would provide a distinct advantage.

33. The undersigned Declarant having been warned that willful false statements and the like are punishable by fine or imprisonment, or both under the provisions of 18 U.S.C. §1001 and may jeopardize the validity of the application or any patent issuing thereon, hereby declares that all statements made of the Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

January 8, 2007
Date

Sinan Tas
Sinan Tas
Declarant